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Expansion of Bacteriocin Activity and Host Range upon Complementation of Two Peptides Encoded within the Lactacin F Operon†

G. E. ALLISON,[1]* C. FREMAUX,[2]‡ AND T. R. KLAENHAMMER[1,2]

Departments of Microbiology[1] and Food Science,[2] Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina 27695-7624

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Lactacin F is a membrane-active bacteriocin produced by *Lactobacillus johnsonii* VPI11088 (Laf$^+$). The genetic determinants encoding lactacin F are organized in a 1-kb polycistronic operon composed of a promoter (P_{laf}), three genes (*lafA*, *lafX*, and ORFZ), and a functional rho-independent transcription terminator. Two Laf$^-$ derivatives of VPI11088, designated NCK64 and NCK65, were characterized. NCK64 contained a frameshift mutation in the *lafA* gene causing premature termination of translation. NCK65 harbored a 10-kb chromosomal deletion covering the *laf* operon. When the *lafA* gene was cloned independently and expressed in NCK65, bacteriocin activity was limited to *L. helveticus* 87, only one of the six known lactacin F-sensitive (Laf^s) indicators. When *lafX* was introduced into NCK65, no bacteriocin activity against any of the sensitive strains was detected. Genetic combination of *lafA* and *lafX*, in *cis* or in *trans*, restored bacteriocin activity against all Laf^s indicators. When two NCK65 clones containing either *lafA* or *lafX* were plated slightly apart on agar plates, fully active lactacin F was present in the intervening area where the two excreted gene products, LafA and LafX, diffused together. The genetic analysis revealed that the interaction of two bacteriocinogenic peptides encoded within the *laf* operon is likely to participate in the formation of poration complexes in the membranes of susceptible bacteria.

Bacteriocins are biologically active peptides, proteins, or protein complexes produced by several bacterial species (14). Lactic acid bacteria (LAB) produce four major classes of bacteriocins that include lantibiotics (class I); small, heat-stable peptides (class II); large proteins (class III); and protein complexes containing essential lipid or carbohydrate moieties (class IV). Bacteriocins in each of these classes have been reviewed (15). The diversity of bacteriocins and their various spectra of activity reflect the broad habitats in which LAB compete, ranging from fermenting food substrates to the intestinal tracts of mammals.

Biochemical and genetic characterization of bacteriocins in LAB has demonstrated that class II peptide bacteriocins are commonly produced (15). The peptides are ribosomally synthesized as inactive precursors with the amino-terminal extension ranging from 18 to 24 residues. The N-terminal end is removed at a consensus Gly-2–Gly-1 · +1 *Xaa* processing site where the Gly-Gly motif is proposed to provoke a β-turn and expose this site to action by a specific protease (19). However, on the basis of site-directed changes at the lactacin F processing site, Fremaux et al. (8) have proposed that the Gly-Gly motif itself, rather than the secondary structure, is vital for recognition and cleavage by the protease. The processing mechanisms and maturation protease appear to be common among LAB, since class II peptides have been characterized in *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Leuconostoc* spp. (11, 15, 18). The structures and hydropathic profiles of these peptides suggest that they could act at the cytoplasmic membrane (27). Indeed, mechanistic studies with lactococcins A (37) and B (39), pediocins JD and PA-1 (3, 4), and lactacin F (1, 3) have shown that the peptides interact with the membranes of susceptible bacteria, cause release of intracellular ions, and deplete the proton motive force. While all act at the membrane, their molecular mechanisms of action may vary considerably and one or more peptides may be involved (8, 26, 36). Consequently, a range of bactericidal to bacteriostatic activities are exerted by different class II peptides (15).

Lactacin F is a prototype class II membrane-active peptide which is produced by *Lactobacillus johnsonii* VPI11088 (16, 17, 23–25), previously named *L. acidophilus* VPI11088 or ATCC 1506 (13) and renamed in accordance with the species classifications of Fujisawa et al. (9). Lactacin F is active against several lactobacilli and *Enterococcus faecalis* (23). The genetic determinants for lactacin F activity are located within an *Eco*RI chromosomal fragment in VPI11088 and consist of three functional open reading frames (ORFs), *lafA*, *lafX*, and ORFZ (8, 16, 17, 25). An upstream promoter (P_{laf}) and a downstream rho-independent terminator flank a 1-kb polycistronic operon that encodes the structural genes for lactacin F (8).

The *lafA* gene was first considered to be the essential structural gene for lactacin F activity, since the N-terminal protein sequence of purified lactacin F corresponded exactly to the deduced sequence within *lafA* (25). However, genetic analysis of the operon revealed that both *lafA* and *lafX* genes are required to express lactacin F activity (2, 8). The peptide deduced from *lafX* possesses most of the molecular features of class II bacteriocins, including a hydrophobic profile, a leader sequence with a consensus processing site, and a C terminal harboring conserved residues (8). This suggests that *lafX* encodes a second bacteriocin-like protein required to exert lactacin F activity via a complementation complex similar to that discovered by Nissen-Meyer et al. (26) for lactococcin G.

* Corresponding author.

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‡ Present address: Institute de biologie moléculaire et d'ingenierie génétique, Faculté de Poitiers, UFR Sciences, 86022 Poitiers Cedex, France.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
L. johnsonii		
VPI11088	NCK88; Laf$^+$ Laf^r; parental strain	VPI[b], 23
88-C	NCK64; VPI11088 derivative; Laf$^-$ (*lafA729*) Laf^r	23
88-4	NCK65; VPI11088 derivative; Laf$^-$ (Δ*laf* operon) Laf^s	23
NCK638	NCK65(pTRK201); *lafA886* Em^r Cm^r	8
NCK639	NCK65(pTRK203); *lafA*$^+$ *lafX1047* Em^r Cm^r	8
NCK642	NCK65(pTRK206); *lafA*$^+$ *lafX*$^+$ Em^r Cm^r	8
NCK659	NCK65(pTRK92); *lafA729 lafX*$^+$ Em^r	This study
NCK660	NCK65(pTRKH2); vector; Em^r	This study
L. fermentum 1750	Laf^s lactacin F indicator	NCDO[c]; 23
L. bulgaricus 1489	Laf^s lactacin F indicator	ATCC[d]; 23
L. lactis 970	Laf^s lactacin F indicator	NCDO; 23
L. delbrueckii subsp. *lactis* ATCC 4797	Laf^s lactacin F indicator	ATCC; 23
E. faecalis ATCC 19433	Laf^s lactacin F indictor	ATCC; 23
L. helveticus 87	Laf^s lactacin F indicator; LafA^s LafA indicator	This study; NCDO; 23
Escherichia coli		
SURE	e14$^-$ (*mcrA*) Δ(*mcrCB-hsdSMR-mrr*) *171 sbcC recB recJ umuC*::Tn*5* (Kan^r) *uvrC supE44 lac gyrA96 relA1 thi-1 endA1* (F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r])	Stratagene
NCK656	*E. coli* SURE(pTRK90)	This study
NCK657	*E. coli* SURE(pTRK91)	This study
NCK658	*E. coli* SURE(pTRK92)	This study
Plasmids		
pBluescript KS+	3.0 kb; Am^r	Stratagene
pTRKH2	6.9 kb; *lacZ* Em^r	28
pTRK90	pBluescript KS+ (*Eco*RV)::0.7-kb *Dra*I-NCK64 PCR product (*lafA729 lafX*$^+$); 3.7 kb	This study
pTRK91	pBluescript KS+ (*Sma*I)::0.7-kb *Dra*I-NCK64 PCR product (*lafA729 lafX*$^+$); 3.7 kb	This study
pTRK92	pTRKH2::0.7-kb *Dra*I-64 PCR product (*lafA729 lafX*$^+$); 7.6 kb; Em^r	This study
pTRK160	pBluescript KS+::2.3-kb *Eco*RI (*lafA*$^+$ *lafX*$^+$ ORFZ$^+$); 5.3 kb	8
pGKV210	4.4 kb; Em^r; promoterless *cat86*	38
pTRK201	pGKV210::0.89-kb *Eco*RI-*Pvu*II pTRK160 (*lafA886*); 5.29 kb; Em^r Cm^r	8
pTRK203	pGKV210::1.05-kb *Eco*RI-*Hae*III pTRK160 (*lafA*$^+$ *lafX1047*); 5.45 kb; Em^r Cm^r	8
pTRK206	pGKV210::0.7-kb *Dra*I pTRK160 (*lafA*$^+$ *lafX*$^+$); 5.1 kb; Em^r Cm^r	8

[a] Laf$^+$, lactacin F producer; Laf$^-$, lactacin F non-producer; Laf^r, lactacin F resistant; Laf^s, lactacin F sensitive; Am^r, ampicillin resistant; Em^r, erythromycin resistant. Cm^r, chloramphenicol resistant.
[b] VPI, Virginia Polytechnic Institute.
[c] NCDO, National Collection of Dairy Organisms.
[d] ATCC, American Type Culture Collection.

In this study, two mutants of *L. johnsonii* VPI11088 deficient in lactacin F production (Laf$^-$) were characterized and then employed as expression hosts to determine how *lafA* and *lafX* are involved in the production of an active bacteriocin. The *lafX* gene product, LafX, was demonstrated to be an essential component of a bactericidal complex that can be formed extracellularly. It is the LafA-LafX complex which is responsible for the spectrum of activity defined previously for lactacin F.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Cultures were maintained and stored at −20°C in 20% glycerol. Lactobacilli were cultured at 37°C in MRS broth (Difco Laboratories, Detroit, Mich.) or MRS containing 3 μg of erythromycin per ml. *E. faecalis* was cultured at 37°C in brain heart infusion (BHI) broth or agar (BBL Microbiology Systems). *Escherichia coli* SURE (Stratagene, La Jolla, Calif.) was propagated at 37°C in LB (31) with 10 μg of tetracycline per ml. When appropriate for clonal selection, ampicillin, erythromycin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and IPTG (isopropyl-β-D-thiogalactopyranoside) were used at concentrations of 50, 200, 50, and 50 μg/ml, respectively (31). Erythromycin-resistant (Em^r) transformants of *E. coli* were selected on BHI agar.

Bacteriocin assays. Bacteriocin activity was assayed by spotting 2.5 or 5 μl of an overnight culture onto MRS agar (1.2%) plates and incubating it under anaerobic gas for 6 to 12 h. The plate was then overlaid with 5.5 ml of MRS agar (1.2%) containing a 1% inoculum of an overnight indicator culture. Overlaid plates were incubated for 12 to 18 h before analysis of results. Activity units (AU) per milliliter of culture supernatant were determined by using the critical-dilution assay described previously (22). Cell-free supernatants were obtained from two centrifugations at 14,000 rpm for 10 min in an Eppendorf 5415 microcentrifuge. For complementation assays, cell-free supernatants from two clones were combined 1:1, serially diluted, and spotted onto overlays inoculated with the indicator.

Chromosomal characterization. Electrophoretic and hybridization patterns of genomic fragments were compared by using pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared and electrophoresed as outlined by Tanskanen et al.

(35), with a 1.2% (wt/vol) agarose gel. *Sma*I digestion of DNA in agarose blocks was done as recommended by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Southern transfer of DNA from agarose gels to Magna-Graph nylon transfer membranes (Micron Separations Inc., Westboro, Mass.) was done as described by Sambrook et al. (31). Labelling of probes and hybridization conditions followed the protocols outlined by the Genius DNA Labelling and Detection Kit (Boehringer Mannheim).

Molecular cloning and transformation. Chromosomal template DNA required for PCR amplification was extracted from NCK64 as outlined by Joerger and Klaenhammer (12). The reagents and amounts are outlined in the GeneAmp Kit (Perkin Elmer-Cetus, Norwalk, Conn.) instructions, and the PCR was conducted under the following conditions: denaturation at 92°C for 15 s, annealing at 50°C for 20 s, and polymerization at 65°C for 2 min, through 30 cycles. Ten microliters of the reaction mixture was electrophoresed on a 0.8% (wt/vol) agarose gel (1× Tris-borate-EDTA buffer, pH 8.3) to examine amplification products. Reaction mixtures were purified by using the GENECLEAN II Kit (Bio 101, La Jolla, Calif.). PCR fragments were digested and ligated (T4 ligase) into vectors under conditions outlined by the manufacturer (Boehringer Mannheim). When necessary, DNA restriction fragments were purified from agarose gels by using the GENECLEAN II Kit (Bio 101). *E. coli* and *Lactobacillus* cells were prepared for electrotransformation as outlined by Dower et al. (5) and Raya et al. (30), respectively. Cells were electroporated in 0.2-cm cuvettes with a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) set at 25 μF, 200 Ω, and 2.5 kV for *E. coli* or 2.1 kV for lactobacilli. After electroporation, cells were incubated in appropriate nonselective media for 1 (*E. coli*) or 2 (lactobacilli) h prior to plating.

DNA sequencing. Plasmid DNA to be sequenced was obtained by proteinase K treatment followed by polyethylene glycol 8000 precipitation of plasmid extracts (31). Sequencing reactions based on the method of Sanger et al. (32) were performed with the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) by following the manufacturer's instructions and using commercial (Stratagene) and specific oligonucleotide primers. DNA and deduced protein sequence analyses were performed with PC/GENE (Intelligenetics, Inc., Mountain View, Calif.).

Purification and SDS-PAGE of LafA and LafX. Cell-free supernatants from 24-h cultures (500 μl) were mixed with an equal volume of butanol and centrifuged for 5 min in an Eppendorf 5415 microcentrifuge at 14,000 rpm in accordance with a protocol developed in our laboratory by J. D. Greene (10). The top butanol fraction was removed and evaporated. The pellet was resuspended in 50 μl of sterile water.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bacteriocin samples was conducted as described by Laemmli (20). The concentrated (10×) cell-free supernatants (15 μl) were combined with 7.5 μl of 4× sample buffer (20) and 7.5 μl of distilled H_2O, boiled for 3 min, loaded onto an SDS–17% PAGE gel, and electrophoresed for 45 min at 200 V in a Mini-PROTEAN II apparatus (Bio-Rad). The gel was washed four times for 30 min each time with sterile picopure water and placed in a petri dish (150 by 15 mm). The gel was overlaid with 25 ml of MRS agar (1.2%) inoculated with the indicator and incubated as described above.

RESULTS

Characterization of chromosomal Laf⁻ mutations in NCK65 and NCK64. VPI11088, NCK65, and NCK64 were compared for gross chromosomal organization by using PFGE. The electrophoretic profiles of the lactacin F producer *L. johnsonii* VPI11088 and its Laf⁻ derivative NCK65 differed (Fig. 1A). A ca. 245-kb band in VPI11088 was absent in NCK65 and replaced by a lower band, estimated at 235 kb. After Southern transfer, the PFGE gel was hybridized with a probe encompassing the *laf* operon (Fig. 2, 2.3-kb *Eco*RI fragment). Three fragments hybridized in VPI11088, including the 245-kb band. Only the two small bands hybridized in NCK65; the probe failed to hybridize to the 235-kb fragment (Fig. 1B). These data indicated that the *laf* operon was located within the 245-kb fragment of VPI11088. The ca. 10-kb deletion, which excluded the *laf* operon from the NCK65 genome, occurred in this fragment. This is consistent with previous observations (25) showing that the 2.3-kb *Eco*RI fragment was deleted in NCK65. The extent of this deletion was not known previously. With this large deletion, NCK65 (Laf⁻) provided a suitable genetic background for functional analysis of individual components of the *laf* operon.

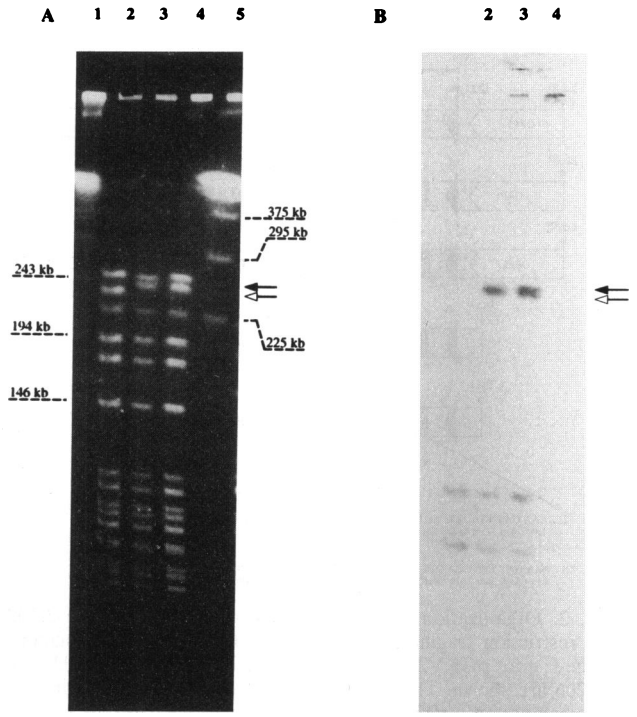

FIG. 1. Chromosomal analyses of *Sma*I digests of *L. johnsonii* VPI11088 (lane 3) and two Laf⁻ mutants, NCK65 (lane 2) and NCK64 (lane 4). (A) PFGE profiles. The molecular weight standards were λ concatemers (lane 1) and yeast chromosomes (lane 5). The solid arrow indicates a ca. 245-kb fragment containing the *laf* operon present in VPI11088 (lane 3) and NCK64 (lane 4). The open arrow indicates a ca. 235-kb fragment present in NCK65. (B) Hybridization profiles of a Southern transfer probed with the 2.3-kb *Eco*RI fragment encoding the *laf* operon.

L. johnsonii VPI11088 and NCK64 (Laf⁻ Laf⁺) exhibited identical PFGE fragmentation and hybridization patterns (Fig. 1A and B), suggesting that the mutation in NCK64 was due to a small deletion or a point mutation. To define the mutation, a region of the chromosome that included P_{laf}, *lafA*, and *lafX* was amplified by PCR. The primers used were complementary to internal regions of ORF1 and ORF2 (Fig. 2). Two separate PCRs and subsequent cloning experiments were performed. The PCR fragments were digested with *Dra*I (Fig. 2) and

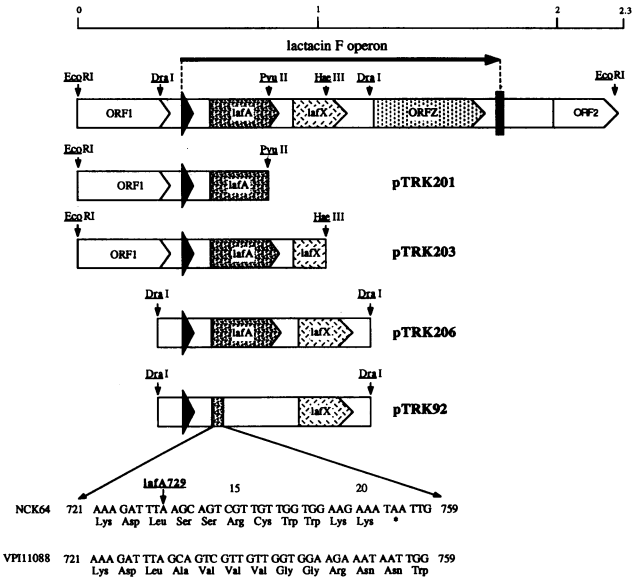

FIG. 2. Organization of the lactacin F operon within the 2.3-kb *Eco*RI restriction fragment cloned from VPI11088. DNA fragments subcloned into pGKV210 to construct pTRK201, pTRK203, and pTRK206 are shown. The DNA fragment from the NCK64 chromosome subcloned into pTRKH2 to construct pTRK92 and the location of the point mutation, *lafA729*, are also shown. ▶, promoter; ■, rho-independent terminator (8, 15).

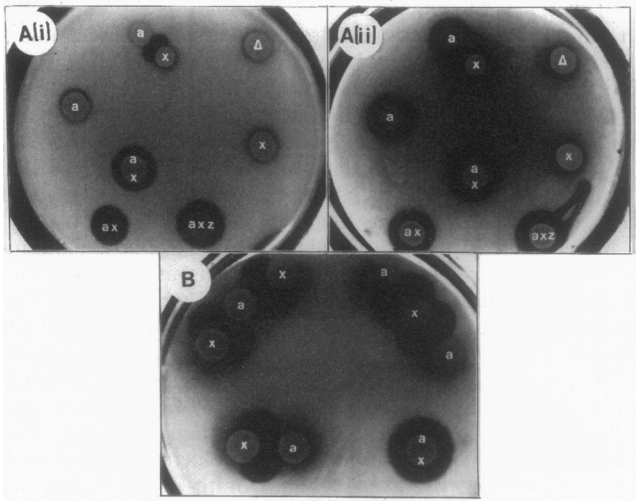

FIG. 3. Evaluation of various *lafA* and *lafX* derivatives for bacteriocin activity. Panels: A(i), assays of activity against *L. delbrueckii* ATCC 4797; A(ii), assays of activity against *L. helveticus* 87; B, assays of activity against *L. delbrueckii* ATCC 4797 done by varying the positions of the LafA and LafX producers. Designations of cultures: ax, NCK642 (*lafA*$^+$ *lafX*$^+$); axz, VPI11088 (*lafA*$^+$ *lafX*$^+$ ORFZ$^+$); a, NCK639 (*lafA*$^+$ *lafX1047*); x, NCK659 (*lafA729 lafX*$^+$); Δ, NCK65 (Δ*laf* operon).

cloned into pBluescript KS+ at either the *Eco*RV or the *Sma*I site to form pTRK90 or pTRK91, respectively. *E. coli* SURE was electroporated with the ligation mixtures, and transformants were selected. Plasmid DNA was extracted and sequenced by using reverse, −40, KS, and SK primers and a synthetic oligonucleotide complementary to a site internal to *lafX*. A total of six clones obtained from both cloning experiments were examined. All contained an additional A at position 729 of the 2.3-kb *Eco*RI *laf* operon fragment, resulting in a frameshift mutation within *lafA* designated *lafA729* (Fig. 2). No other changes were observed over the 664 bp sequenced. This frameshift causes a missense mutation in residue 13 of the 75-amino-acid precursor. Nine codons downstream of the mutation site, a stop codon is generated that would cause premature termination of LafA translation. This mutation does not alter the reading sequence of P_{laf}, *lafX*, or its ribosomal binding site. Therefore, the Laf$^-$ phenotype of NCK64 appeared to result from its failure to produce the LafA peptide.

Complementation of LafA and LafX. The phenotype-genotype relationship in NCK64 indicated that *lafA* is required for bacteriocin activity. The *lafX* gene has also been reported to be essential, since its physical disruption destroys lactacin F activity (8). To determine the interaction between *lafA* and *lafX*, the two genes were cloned individually or together by using either promoter-probe vector pGKV210 (38) or pTRKH2 (28) and introduced into NCK65.

The *Eco*RI-*Hae*III fragment containing P_{laf} and *lafA* from VPI11088 was cloned previously into pGKV210 to form pTRK203 (Fig. 2; reference 8). This plasmid, containing *lafA* and a truncated *lafX* gene, designated *lafX1047*, was electroporated into NCK65 to form NCK639. NCK639 did not exert inhibitory activity towards *L. delbrueckii* ATCC 4797 (Fig. 3A[i]). The NCK639 culture was further evaluated for inhibition of the spectrum of known lactacin F indicators (Laf^s) (23). NCK639 did not inhibit Laf^s strains *L. fermentum* 1750, *L. delbrueckii* subsp. *bulgaricus* 1489, *L. delbrueckii* subsp. *lactis* 970, and *E. faecalis* ATCC 19433 (data not shown). However, NCK639 (*lafA*$^+$ *lafX1047*) did inhibit *L. helveticus* 87 (Fig. 3A[ii]). Therefore, LafA was excreted from NCK639 and exerted activity against one of the six lactacin F indicators.

It was determined previously that both *lafA* and *lafX* are essential if constructions in NCK65 are to exert inhibitory activity against *L. delbrueckii* ATCC 4797 (8). In this study, NCK642 [NCK65(pTRK206); Fig. 2], containing the P_{laf} *lafA lafX* cassette, was further evaluated for inhibitory activity against all six lactacin F indicators. NCK642 (*lafA*$^+$ *lafX*$^+$) inhibited all of the Laf^s strains. Results obtained with the two most sensitive indicators, *L. delbrueckii* ATCC 4797 and *L. helveticus* 87, are shown in Fig. 3A[i] and A[ii], respectively. Therefore, although LafA exerted some bactericidal action by itself, both *lafA* and *lafX* contributed to the overall inhibitory spectrum defined for lactacin F.

A *lafA729 lafX*$^+$ construction was cloned into NCK65. The *Dra*I fragment PCR amplified from the NCK64 chromosome (see above) was excised from pTRK90 (Table 1) at the *Sal*I-*Bam*HI sites and ligated into the *Sal*I-*Bgl*II sites of pTRKH2, a high-copy-number vector containing the pAMβ1 origin from pIL253 (28). *E. coli* SURE was electroporated with the ligation mixture, and clones containing the recombinant plasmid, designated pTRK92 (Fig. 2), were selected (28). To examine the role of *lafX* in lactacin F activity, pTRK92 was introduced into *L. johnsonii* NCK65 by electroporation to create NCK659 (*lafA729 lafX*$^+$). NCK659 did not inhibit any of the lactacin F indicators. The results obtained with *L. delbrueckii* ATCC 4797 and *L. helveticus* 87 are shown in Fig. 3A[i] and A[ii]. These data suggest that the *lafX* product was either not excreted or not bactericidal on its own.

A bacteriocin agar complementation assay was developed to investigate whether the *lafX* gene product was excreted and could function extracellularly to promote lactacin F activity.

TABLE 2. Determination of AU per milliliter

Strain/ complement[a]	Strain or strain/complement genotype(s)	Activity (AU/ml)[b] against:	
		L. delbrueckii ATCC 4797	*L. helveticus* 87
NCK64	*lafA729 lafX*$^+$	<100	<100
NCK65	Δ*laf* operon	<100	<100
NCK64/NCK65	*lafA729 lafX*$^+$/Δ*laf* operon	<100	<100
NCK638	*lafA886*	<100	<100
NCK660	Δ*laf* operon	<100	<100
NCK638/NCK660	*lafA886*/Δ*laf* operon	<100	<100
NCK639	*lafA*$^+$ *lafX1047*	<100	400
NCK659	*lafA729 lafX*$^+$	<100	<100
NCK639/NCK659	*lafA*$^+$ *lafX1047*/*lafA729 lafX*$^+$	800	3,200
NCK639/NCK660	*lafA*$^+$ *lafX1047*/Δ*laf* operon	<100	400
NCK638/NCK659	*lafA886*/*lafA729 lafX*$^+$	<100	<100

[a] For complementation assays, separate samples from the supernatants of the strains indicated were mixed in a 1:1 ratio.

[b] Each value represents an average of several trials with different supernatant samples. Averages were computed and rounded to the nearest dilution as outlined by Mayr-Harting et al. (22).

Cells of NCK659 (*lafA729 lafX*$^+$) were spotted onto MRS agar near cells of NCK639 (LafA$^+$). After 6 to 12 h of incubation, the plates were overlaid with either *L. delbrueckii* ATCC 4797 or *L. helveticus* 87. Figure 3A[i] and B show that bacteriocin activity against ATCC 4797 was recovered in the region between the two clones. A zone of *L. helveticus* 87 inhibition also occurred around NCK659 (*lafA729 lafX*$^+$) upon interaction with LafA produced by NCK639 (Fig. 3A[ii]). Additional agar complementation assays demonstrated bacteriocin activity against all six lactacin F indicators in a zone between NCK659 and NCK639 cultures where LafA and LafX diffused together (data not shown). If NCK659 and NCK639 cultures were mixed 1:1 and plated, a large zone of inhibition against all indicators was observed around the *L. johnsonii* "mixed" culture (Fig. 3A[i] and A[ii]). Therefore, LafA and LafX can be individually excreted and extracellularly complement one another to exert a full spectrum of lactacin F activity.

The AU per milliliter of cell-free supernatants of LafA and LafX producers, singly and in mixtures thereof, were determined against the two sensitive indicators, ATCC 4797 and 87 (Table 2). NCK639 (LafA$^+$) cell-free supernatant was active toward 87 at a level of 400 AU/ml; NCK659 (LafX$^+$) showed no activity against 87. When NCK639 and NCK659 supernatants were combined in a 1:1 ratio, activity against 87 was increased eight times, to a level of 3,200 AU/ml. Neither supernatant from NCK639 nor that from NCK659 was active against ATCC 4797 independently, but when they were combined, the mixture exerted activity against *L. delbrueckii* ATCC 4797 at a level of 800 AU/ml.

Bactericidal activity of LafX. The predicted amino acid sequence of LafX exhibits characteristics that are consistent with class II bacteriocins defined in LAB, notably, a putative Gly-Gly processing motif and a hydrophobic character (8). However, conventional bacteriocin assays failed to establish any bacteriocin activity for LafX alone. Efforts were made, therefore, to concentrate LafX and further examine it for activity. From cell-free supernatants, samples of LafX (from NCK659), LafA (from NCK639), and LafA plus LafX (from NCK642) were concentrated 10-fold through a butanol extraction procedure (10). The concentrated samples were electrophoresed on denaturing gels and examined for bacteriocin activity by overlaying the gel with a lawn of *L. helveticus* 87 (Fig. 4). Zones of inhibition occurred for each sample at similar positions in the gel consistent with the migration behavior expected of low-molecular-weight peptides. The positive control (lactacin F, purified from VPI11088) exhibited activity (Fig. 4, lane 1), whereas the negative control, NCK660, did not (data not shown). These data demonstrated that both peptides are bacteriocinogenic, with LafX capable of exerting some detectable activity when highly concentrated.

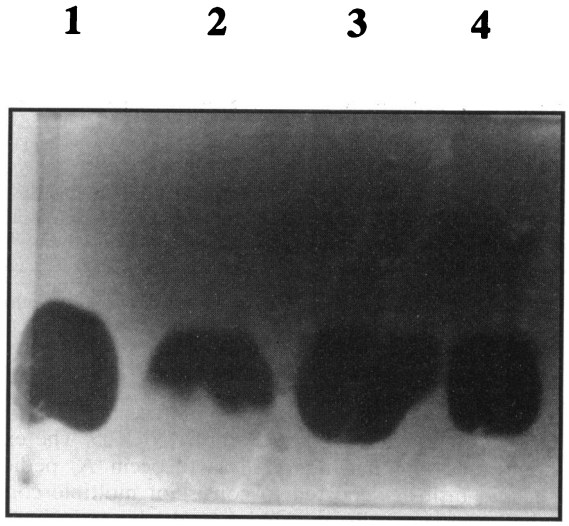

FIG. 4. Bacteriocin activity localized in protein gels after SDS-PAGE of 10×-concentrated samples of LafA and/or LafX purified from cell-free culture supernatants of the appropriate producer strain. The gels were overlaid with *L. helveticus* 87. Lanes: 1, *L. johnsonii* VPI11088 (parental lactacin F producer); 2, NCK659 (LafX$^+$); 3, NCK639 (LafA$^+$); 4, NCK642 (LafA$^+$ LafX$^+$). Samples purified from NCK660 (NCK65 harboring the vector pTRKH2) failed to exhibit activity (data not shown).

DISCUSSION

The genetic analyses presented in this study show that "lactacin F activity" is defined by functional *lafA* and *lafX* genes located adjacent to one another in the lactacin F operon. When *lafA* is expressed, the excreted LafA product is bactericidal towards one indicator, *L. helveticus* 87. With addition of the *lafX* gene product, activity against 87 increases and the inhibitory spectrum expands to include other known lactacin F indicators, *L. delbrueckii* 4797, *L. delbrueckii* subsp. *bulgaricus* 1489, *L. delbrueckii* subsp. *lactis* 970, and *E. faecalis* ATCC 19433. LafX is bactericidal toward *L. helveticus* 87 only when highly concentrated on SDS-PAGE gels. LafX shares many characteristics with its complementary bacteriocinogenic peptide, LafA, including size, hydrophobicity, and a putative posttranslational cleavage site (8).

Complementation between LafA and LafX has been demonstrated by cloning respective genes in *cis* (NCK642 [Table 1]) or *trans* (NCK466 [8]) and extracellularly when the individual genes are cloned and expressed by NCK65. NCK65 was chosen as the host for functional analysis of *lafA* and *lafX* because it was determined to harbor a deletion of ca. 10 kb which excludes the *laf* operon from its genome; therefore, analysis of NCK65 clones would assign phenotypes to respective genes without the influence of other components within the *laf* operon.

TABLE 3. Sequence analysis of antibacterial peptides

Peptide	Amino acid sequence of mature peptide[a]	Reference(s)
Cecropin A	KWKLFKKIEKVGQNIRDGIIKA **GP** AVAVVGQATQIAK	6
Lactococcin M	IRGTGKGLAAAMVSGAAMGGAIGAFG **GP** VGAIMGAWGGAVGGAMKYSI	29, 36
Lactococcin N	MKKDEANTFKEYSSSFAIVTDEELENING	29, 36
	SGSIWGAIAGGAVKGAIAASWTG **NP** VGIGMSALGGAVLGGVTYARPVH	
Lactococcin Gβ	KKWGWLAWVDPAYEFIKGFGKGAIKE **GN** KDKWKNI	26, 33
Lactococcin $G\alpha_1$	GTWDDIGQGIGRVAYWVGKAMGNMSDV **N** QASRINRKKKH	26, 33
LafX	NRWGDTVLSAASGAGTGIKACKSF **GP** WGMAICGVGGAAIGGYFGYTHN	29
LafA	RNNWQTNVGGAVGSAMIGATVGGTIC **GP** ACAVAGAHYLPILWTGVTAATGGFGKIRK	29

[a] Underlining indicates the position of the predicted transmembrane helix, and letters in boldface are areas of conserved amino acids which may have a hinge function (6).

With a deletion of this size, it is interesting that both peptides were excreted and processed by NCK65. The excretion and processing machinery of lactococcin A, pediocin, subtilin, and nisin is reported to consist of multiple components that are genetically encoded near the bacteriocin structural genes (7, 18, 21, 34). Many of these "secretion" genes share homology to the HlyB family of transport proteins that are ATP dependent and signal sequence independent (7, 18, 34). The location of the deletion in NCK65 is unknown, but its ability to express LafA and LafX may be explained by the chromosomal analysis, which suggests that the operon and surrounding sequences are copied elsewhere in the genome. Three fragments in NCK88 hybridized when probed with the 2.3-kb *Eco*RI fragment; two of these genomic fragments in NCK65 continued to hybridize despite the 10-kb deletion, suggesting that the processing machinery could be duplicated in other regions of the chromosome. If the deletion in NCK65 did exclude bacteriocin-specific processing and excretion genes, it is possible that lactacin F is just one of many products processed and secreted by a nonspecific housekeeping system. These possibilities have been discussed previously (18) and may explain the reduced bacteriocin production of NCK65 clones (8, 25). Further mapping of the NCK65 deletion and characterization of up- and downstream sequences from the wild-type *laf* operon may elucidate those genes required for processing and secretion.

The finding that both *lafA* and *lafX* gene products are essential for lactacin F activity is ambiguous relative to results reported previously (24, 25). When purified material with lactacin F activity was subjected to N-terminal sequence analysis, a single sequence of 25 amino acids was determined (24). By using this sequence, a 63-mer oligonucleotide probe was deduced and used to clone the structural *lafA* gene. The LafX peptide was not identified by the biochemical analysis, even though the indicator used routinely in the purification process of the previous study (24) was shown here to be sensitive only to both peptides. This activity indicates that LafA and LafX were copurified. The high-pressure liquid chromatography chromatogram (24) showed that a broad-based peak was correlated with lactacin F activity, suggestive of a heterogeneous sample. Perhaps the LafX peptide is blocked or unavailable for Edman degradation. Further biochemical analysis of the individual peptides and the nature of their interaction is required.

Other bacteriocin systems defined by two heterogeneous components have also been reported. Lactococcin M (LcnM/N) activity requires the presence of functional *lcnM* and *lcnN* genes (36). Lactococcin G (LcnG) activity results from the complementary action of β and α peptides (26). LcnG and many other antibacterial peptides, including the cecropins, have been proposed to form a pore in the cytoplasmic membrane through the barrel stave mechanism described by Ojcius and Young (27). Three distinct steps are involved in pore formation: monomers in solution interact with the cytoplasmic membrane, insert themselves into the membrane, and aggregate like barrel staves surrounding a central pore which allows leakage of intracellular components. The only structural motif common to monomers are regions of at least 10 or 20 residues with the potential to form amphipathic β-sheets or α-helices, respectively. The monomer may exist in these conformations in solution or undergo a conformational change from a random coil to a β-sheet or α-helix upon interaction with the cytoplasmic membrane. Indeed, mechanistic studies have demonstrated that lactacin F interacts with the membranes of *L. delbrueckii* ATCC 4797 and *E. faecalis* ATCC 19433 to create ion channels and deplete the proton motive force (1, 3).

The amino acid sequences of LcnM, LcnN, LcnGβ, $LcnG\alpha_1$, LafA, and LafX are shown in Table 3. Cecropin A has been included because its secondary structure (helix-bend-helix) has been determined and a model for pore formation has been proposed (6). By analyzing the amino acid sequences by the method of Rao and Argos (29), LcnM, LcnN, and LafA are predicted to have motifs similar to that of cecropin A. LcnGβ, $LcnG\alpha_1$, and LafX are predicted to form a single helix. While use of this method for structure predictions demonstrates interesting similarities among two-component bacteriocin peptides and cecropin A, there are limitations in these projections: the predicted helices vary from perfect amphipathicity, and LafA and LafX contain a high proportion of helix-breaking residues within the putative helical regions. Further experimental data on the secondary structures are required before the significance of the similarities between the two component bacteriocin peptides may be addressed.

The mode of action of many bacteriocins produced by LAB remains to be determined. The lactacin F poration complex is the first two-component system for which genetic, biochemical, and mechanistic data are available. Future studies can now identify motifs in each peptide that are important for oligomerization and/or receptor-membrane interaction. The barrel stave mechanism is likely to be a common theme among bacteriocins produced by several strains of bacteria. Identification of certain motifs essential to this three-step process could lead to the engineering of broad-spectrum bacteriocins for dairy foods.

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